Faxed to Greaves Brewster & copied to dstl on 11-4-02 C Smith

Please file C Smith

# PATENT COOPERATION TREATY

From the:
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:
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16 APR 2002
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# PCT

## WRITTEN OPINION

(PCT Rule 66)

| | |
|---|---|
| Date of mailing *(day/month/year)* | 11.04.2002 |
| Applicant's or agent's file reference: P1247/WOD | **REPLY DUE** **within 0 month(s) and 15 days** from the above date of mailing |

| International application No. | International filing date *(day/month/year)* | Priority date *(day/month/year)* |
|---|---|---|
| PCT/GB00/04133 | 26/10/2000 | 26/10/1999 |

International Patent Classification (IPC) or both national classification and IPC
C12N15/53

Applicant
THE SECRETARY OF STATE FOR DEFENCE et al.

1. This written opinion is the **first** drawn up by this International Preliminary Examining Authority.

2. This opinion contains indications relating to the following items:

| | | |
|---|---|---|
| I | ☒ | Basis of the opinion |
| II | ☐ | Priority |
| III | ☐ | Non-establishment of opinion with regard to novelty, inventive step and industrial applicability |
| IV | ☐ | Lack of unity of invention |
| V | ☒ | Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement |
| VI | ☐ | Certain document cited |
| VII | ☐ | Certain defects in the international application |
| VIII | ☐ | Certain observations on the international application |

3. The applicant is hereby **invited to reply** to this opinion.

**When?** See the time limit indicated above. The applicant may, before the expiration of that time limit, request this Authority to grant an extension, see Rule 66.2(d).

**How?** By submitting a written reply, accompanied, where appropriate, by amendments, according to Rule 66.3. For the form and the language of the amendments, see Rules 66.8 and 66.9.

**Also:** For an additional opportunity to submit amendments, see Rule 66.4.
For the examiner's obligation to consider amendments and/or arguments, see Rule 66.4 bis.
For an informal communication with the examiner, see Rule 66.6.

**If no reply is filed**, the international preliminary examination report will be established on the basis of this opinion.

4. The final date by which the international preliminary examination report must be established according to Rule 69.2 is: 26/02/2002.

Name and mailing address of the international preliminary examining authority:
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## I. Basis of the opinion

1. With regard to the **elements** of the international application (Replacement *sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed"*):

**Description, pages:**

1-43 as originally filed

**Claims, No.:**

1-25 as originally filed

**Drawings, sheets:**

1/24-24/24 as originally filed

**Sequence listing part of the description, pages:**

1-8, as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☒ contained in the international application in written form.
- ☒ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   ☐ the description, pages:
   ☐ the claims, Nos.:
   ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   *(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)*

6. Additional observations, if necessary:

**V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

1. Statement

| | | |
|---|---|---|
| Novelty (N) | Claims | 1, 2, 5, 6, 11(a,e), 12, 14-16, 18-23 |
| Inventive step (IS) | Claims | 2-4, 10, 17 |
| Industrial applicability (IA) | Claims | |

2. Citations and explanations
   **see separate sheet**

**Re Item V**

**Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

1). Reference is made to the following documents:

D1: WO 99 14336 A (PROMEGA CORPORATION (US); WOOD KEITH V.; HALL MARY P.) 25 March 1999;

D2: LI YE et al.: ' Cloning and sequencing of a cDNA for firefly luciferase from *Photuris pennsylvanica* ' BIOCHIMICA ET BIOPHYSICA ACTA, vol. 1339, 25 April 1997, pages 39-52, XP000909154.

2.1). Document D1 describes the *Photuris pennsylvanica* luciferase mutants " Luc*78-0B10* " and " Luc*90-1B5* " with increased thermostability (D1: page 13 and tables 1 and 2). The amino acid sequence of these mutants is disclosed in D1 (D1: page 4 and figures 36 and 43). The amino acid sequence of the corresponding wild-type enzyme is also disclosed (D1: page 3 and figure 45). In the wild-type amino acid sequence, it can be seen that the amino acid residue corresponding to residue 357 in *Photinus pyralis* luciferase, is the Valine residue in the stretch PDTD**V**RPGS. This correspondence is indicated in the sequence alignment of figure 2 of document D2, to which present application refers for the identification of residues corresponding to a certain position in the *Photinus pyralis* luciferase amino acid sequence. This wild-type Valine residue is substituted in the mutants " Luc*78-0B10* " and " Luc*90-1B5* " with an Alanine (D1: figures 36 and 43). Moreover, in the amino acid sequences of mutants " Luc*78-0B10* " and " Luc*90-1B5* ", the following additional mutations can be observed:

a) the Aspartic Acid residue in the stretch LITP**D**TDVR, is substituted with a Lysine. This Aspartic Acid residue is the one corresponding to position 354 in *Photinus pyralis* luciferase, as indicated in the sequence alignment of D2 already mentioned (D2: figure 2);

b) the Phenylalanine residue in the stretch LMAF**F**AKSA, is substituted with a Leucine. This Phenylalanine residue is the one corresponding to position 295 in *Photinus pyralis* luciferase.

In addition to this, document D1 discloses the nucleic acid sequences coding for said mutants " Luc*78-0B10* " and " Luc*90-1B5* " (D1: figures 32 and 42, respectively). A *Bam*HI restriction site (GGATCC) is present at the beginning of both sequences. This restriction site is not present in the nucleic acid sequence encoding the luciferase, as originally isolated from *Photuris pennsylvanica* (as indicated in figure 1 of D2). Therefore, the nucleic acid sequences disclosed in figures 32 and 42 of D1 is embraced by the scope of claim 15.
An expression vector comprising said nucleic acid, *Escherichia coli* cells transformed with said vector, a method of producing said luciferase mutants, which method comprises culturing said *Escherichia coli* cell are described in D1 (D1: pages 2-3). This description is considered to be an enabling disclosure for the subject-matter of claims 18-20 because at the priority date of present application, expression vectors, host cells and corresponding methods for the production of recombinant proteins were tools very well known to the person skilled in the art and currently used in the technical field.
The use of said luciferase mutants in a bioluminescent assay and a kit comprising said mutants and luciferin are also described in D1 (D1: pages 16-20).

2.2). The subject-matter of claims **1**, **2**, **5, 6**, **11(a,e)**, **12**, **14-16** and **18-23** (for claim 2, insofar as it refers to a mutant *Photuris pennsylvanica* luciferase) is therefore not novel (Article 33(2) PCT).

3.1). Document D1, which is considered to represent the most relevant state of the art, discloses *Photuris pennsylvanica* luciferase mutants with increased thermostability, as already commented under previous point 2.1). The subject-matter of claims 2-4, 10 and 17 differs in that mutants of luciferases from species other than *Photuris pennsylvanica* are concerned.

3.2). The problem to be solved by the present invention may therefore be regarded as the provision of further luciferases with increased thermostability

3.3). The solution proposed in claims **2-4**, **10** and **17** of the present application cannot be considered as involving an inventive step (Article 33(3) PCT) for the following reasons: document D1 recites on pages 6-7 that the overall three-dimensional structure of all beetle luciferases is quite similar and that high thermostability can

be achieved for other beetle luciferases by methods similar to the one disclosed in D1. Moreover, on page 8 of D1 it is stated that, since all beetle luciferases belong to the same structural class, they also share in the same pool of potentially stabilizing mutations. In addition to this, on page 9 of D1 it is stated that " similar results were achieved using another beetle luciferase from *Pyrophorus plagiophthalamus* ".

These statements can be considered as a suggestion that the same mutations found to lead to increased thermostability in *Photuris pennsylvanica*, as disclosed in D1, may also be applied to luciferases from related species, as the ones disclosed in figure 17 of D1 and whose amino acid sequence is aligned in figure 19 of D1. Therefore, the disclosure of document D1 would be considered by the person skilled in the art as an incentive to mutate other beetle luciferases, in the positions corresponding to the ones mutated in the thermostable mutants " Luc*78-0B10* " and " Luc*90-1B5* ".

Consequently, starting from the description of D1, a person skilled in the art would have arrived at the obtainment of mutant luciferases which fall within the scope of claims 2-4, 10 and 17 with a reasonable expectation of success and without using his inventive skill, requiring nothing extraordinary all being a matter of technical convenience.

The following remarks are done with respect to Article 6, Rule 6 PCT.

4.1). Claim 4 is not supported by the description as required by Article 6 PCT, as its scope is broader than justified by the description. The reasons therefor are the following: the subject-matter of claim 4 relates to a recombinant protein having luciferase activity, wherein in the sequence of the enzyme, the amino acid residue corresponding to residue " 357 " in *Photinus pyralis* luciferase is other than aspartic acid or glutamic acid. The claim covers all the substitutions in position " 357 " of *Photinus pyralis* luciferase (or corresponding positions in the other luciferases) with **any amino acid residue other than aspartic acid or glutamic acid**, as can be interpreted by the wording of the claim. Present application, however, provides disclosure within the meaning of Article 5 PCT and support within the meaning of Article 6 PCT for only 12 out of the 18 possible substitutions. In fact, in the example 2, tables 2 and 3 of present application, the following mutants are disclosed: D357K, D357R, D357S, D357N, D357V, D357T,

D357L, D357I, D357W, D357F, D357Y. Moreover, in table 5, on page 37-38 of present application, double mutants having the mutation D357M are disclosed. The thermostability of these mutants is disclosed in tables 2, 3 and 6 of present application. From table 6, it can be seen that the mutant D357K (identified as Enzyme No. 25) is not thermostable (0.1% activity remaining after incubation at 45° for 4 minutes, as compared to 0.05% activity of the wild-type enzyme tested at the same conditions). The change of wavelength of emitting light of some of the mutants is disclosed in tables 4 and 5 of present application. Again, in table 4, it can be seen that mutant D357K shows a deviation from wild-type luciferase of only 2nm, in terms of wavelength of emitted light. This difference is considered not to be high enough to distinguish the scope of said mutant from the one of the wild-type luciferase.
Therefore it should be concluded that, in the light of the fact that not all the D357 mutants disclosed, possess the desired capability of emitting light at a different wavelength and/or has enhanced thermostability as compared to the corresponding wild-type luciferase, mutations at position " 357 " of *Photinus pyralis* luciferase cannot be generalized in terms of the effect obtained. Consequently, there exist serious doubts that **all** the mutations not disclosed might also be embraced by the scope of claim 4.

4.2). In addition to this, the embodiment of the invention concerning mutant D357K does not fall within the scope of the claims for the reasons already outlined in previous point 3.1). This inconsistency between the claims and the description leads to doubt concerning the matter for which protection is sought, thereby rendering the claims unclear (Article 6 PCT).

4.3). Claim 1 does not meet the requirements of Article 6 PCT in that the matter for which protection is sought is not clearly defined. The following functional statement: " ability to emit light at a different wavelength and/or possession of enhanced thermostability, as compared to the corresponding wild-type luciferase " does not enable the skilled person to determine which technical features are necessary to perform the stated functions. In fact, for the reasons already outlined under previous point 3.1), the presence of a mutation in the position corresponding to position " 357 " of *Photinus pyralis* luciferase is not sufficient to unambiguously define the activity of the luciferases concerned.

5). In claims 2, 4 and 5 and in several parts of the description it is referred, among other luciferases, to a luciferase from *Hotaria paroula*, *Pyrocoelia* ***nayako*** or ***Photinus*** *pennsylvanica*. Said luciferases should be interpreted as being the ones disclosed in document D2, since in page 4, present application makes reference to said document in order to identify the corresponding residues of the luciferases concerned. However, in document D2 it is referred to *Hotaria parvula*, *Pyrocoelia* ***miyako*** and ***Photuris*** *pennsylvanica* luciferases (D2: table 1). This inconsistency between the organisms indicated in the claims and the description (with reference to document D2) leads to doubt concerning the matter for which protection is sought, thereby rendering the claims unclear (Article 6 PCT).

6.1). In claim 11(f), the residue 16 of *Luciola cruciata* or *Luciola lateralis* luciferases are indicated as being corresponding to residue 14 of *Photinus pyralis* luciferase. In the description on page 9, however, is indicated that residue **17** of *Luciola cruciata* or *Luciola lateralis* luciferases is corresponding to said residue 14 of *Photinus pyralis* luciferase.
Analogously, In claim 11(g), residue 37 of *Luciola cruciata* or *Luciola lateralis* luciferases are indicated as being corresponding to residue 35 of *Photinus pyralis* luciferase. In the description on page 9, however, is indicated that residue **38** of *Luciola cruciata* or *Luciola lateralis* luciferases is corresponding to said residue 35 of *Photinus pyralis* luciferase.
Again In claim 11(h), residue 106 of *Luciola cruciata* or *Luciola lateralis* luciferases are indicated as being corresponding to residue 105 of *Photinus pyralis* luciferase. In the description on page 9, however, is indicated that residue **107** of *Luciola cruciata* or *Luciola lateralis* luciferases is corresponding to said residue 105 of *Photinus pyralis* luciferase.
This inconsistency between the corresponding positions indicated in the claims and the description leads to doubt concerning the matter for which protection is sought, thereby rendering the claims unclear (Article 6 PCT).

6.2). In page 5 of present application it is cited that in some forms of the luciferase of ***Photinus*** *pennsylvanica*, the residue corresponding to residue 357 of *Photinus pyralis* luciferase is the residue Valine in position 355. Having regard to the sequence alignment in figure 2 of document D2, and to the amino acid sequence of ***Photuris*** *pennsylvanica* luciferase disclosed in figure 1 of document D2, it

seems that the corresponding residue is indeed the Valine residue on position **356** of ***Photuris*** *pennsylvanica* luciferase.

6.3). In page 9, lines 35-36 of present application it is not clear how the amino acid residue 105 of *Photinus pyralis* luciferase may correspond to both residues 107 and 108 in *Luciola lateralis* luciferase.

6.4). In page 11 lines 34-36, it is indicated, as an additional mutation, the presence of a residue other than Phenylalanine in position 16 of the luciferases from *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis*. Having regard to the comments already made in previous point 5.1), it seems that position 16 of the luciferase from *Luciola mingrelica* (actually consisting of a Phenylalanine residue in the wild-type luciferase) corresponds to position **17** of the luciferases from *Luciola cruciata* or *Luciola lateralis* (also consisting of a Phenylalanine residue in the wild-type luciferase). The same remark applies also to the passage in page 15, lines 11-19.

6.5). In page 12 lines 4-6, it is indicated, as an additional mutation, the presence of a residue other than Glycine in position 106 of the luciferases from *Luciola mingrelica*, *Luciola cruciata* or *Luciola lateralis*. Having regard to the comments already made in previous point 5.1), it seems that position 106 of the luciferase from *Luciola mingrelica* (actually consisting of a Glycine residue in the wild-type luciferase) corresponds to position **107** of the luciferases from *Luciola cruciata* or *Luciola lateralis* (also consisting of a Glycine residue in the wild-type luciferase). The same remark applies also to the passage in page 16, lines 5-17.

6.6). Having regard to the remarks in previous points 5.1-5.5), it seems that identification of " corresponding residues " only by means of the relative position of the residue may very easily lead to ambiguities. The Applicant is therefore requested to identify said corresponding residues by means of their technical features (i.e. the amino acid residue present in the wild type enzyme, in the context of the stretch of amino acids comprising said residue).

7). In order to rule out any ambiguity, in claim 7, the amino acids indicated as uncharged polar should be explicitly defined.

The Applicant is requested to file amendments by way of replacement pages in the manner stipulated by Rule 66.8(a) PCT. In particular, fair copies of the amendments should be filed preferably in triplicate.

In order to facilitate the examination of the conformity of the amended application with the requirements of Article 34(2)(b) PCT, the Applicant is requested to clearly identify the amendments carried out, irrespective of whether they concern amendments by addition, replacement or deletion, and to indicate the passages of the application as filed on which these amendments are based.
If the Applicant regards it as appropriate these indications could be submitted in handwritten form on a copy of the relevant parts of the application as filed.

Moreover, the Applicant's attention is drawn to the fact that, as a consequence of Rule 66.8(a) PCT the examiner is not permitted to carry out any amendments under the PCT procedure, however minor these may be.